

A toolkit for GFP-mediated tissue-specific protein degradation in *C. elegans*

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ABSTRACT

Proteins that are essential for embryo production, cell division and early embryonic events are frequently reused later in embryogenesis, during organismal development or in the adult. Examining protein function across these different biological contexts requires tissue-specific perturbation. Here, we describe a method that uses expression of a fusion between a GFP-targeting nanobody and a SOCS-box containing ubiquitin ligase adaptor to target GFP-tagged proteins for degradation. When combined with endogenous locus GFP tagging by CRISPR-Cas9 or with rescue of a null mutant with a GFP fusion, this approach enables routine and efficient tissue-specific protein ablation. We show that this approach works in multiple tissues – the epidermis, intestine, body wall muscle, ciliated sensory neurons and touch receptor neurons – where it recapitulates expected loss-of-function mutant phenotypes. The transgene toolkit and the strain set described here will complement existing approaches to enable routine analysis of the tissue-specific roles of *C. elegans* proteins.

KEY WORDS: Protein degradation, *C. elegans*, ZIF-1, GFP nanobody, vhhGFP4

INTRODUCTION

Techniques for disrupting protein function in specific tissues or at particular points in development are enabling detailed analysis of developmental mechanisms. To enable tissue-specific perturbation of gene function in *C. elegans*, methods for Cre-lox-, FLP-FRT- and CRISPR-Cas9-based gene knockouts, as well as tissue-specific RNAi have been described previously (Hubbard, 2014; Qadota et al., 2007; Shen et al., 2014). However, the utility of DNA/RNA-editing approaches can be limited by perdurance of the target protein following excision, which can delay the manifestation of phenotypes.

An alternative approach that circumvents this problem is to directly target proteins for degradation in specific tissues. One method for achieving this that was recently adapted for *C. elegans* (Zhang et al., 2015) is based on transplanting the auxin-induced protein degradation system from plants (Holland et al., 2012;

Nishimura et al., 2009). In this system, addition of the small molecule auxin activates a plant-specific F-box protein, TIR1, that serves as a substrate recognition component for a Skp1–Cullin–F-box (SCF) E3 ubiquitin ligase. Active TIR1 targets proteins containing a specific degron sequence. However, a robust genetically encoded method that does not require a small molecule would also be useful, as the exposure kinetics and dose of small molecules in *C. elegans* is limited by barriers such as the cuticle and the eggshell. To this end, a method was developed that takes advantage of an endogenous *C. elegans* protein degradation system. With this approach, target proteins are tagged with a 36 amino acid degron sequence (ZF1); the SOCS-box adaptor protein ZIF-1, which targets ZF1-containing proteins for proteasomal degradation, is expressed in the target tissue (Armenti et al., 2014). However, a limitation of this system is that ZIF-1 plays an essential role during early embryogenesis (DeRenzo et al., 2003; Reese et al., 2000). Thus, proteins tagged with the ZF1 degron are degraded during embryogenesis as well as in the target tissue, which is problematic for analysis of proteins that function during embryogenesis as well as at later developmental stages. More recently, a bacterial sortase-based protein ligation approach was also used to achieve targeted protein degradation in *C. elegans* (Wu et al., 2017). This approach requires tagging the target protein with a small five amino acid tag and expressing a tagged F-box along with a modified sortase.

Here, we have developed a new system that combines potent ZIF-1-mediated protein degradation (Armenti et al., 2014) with the previously described deGradFP approach (Caussinus et al., 2012). In deGradFP, a GFP nanobody is fused to a F-box protein to degrade GFP-tagged proteins. As neither the originally described F-box adaptor nor the *C. elegans* LIN-23 F-box works in *C. elegans* (S.W. and K.O., unpublished), we fused the GFP nanobody to ZIF-1. We show that expression of this fusion enables efficient depletion of GFP-tagged proteins across multiple tissues. In conjunction with GFP tagging at endogenous loci using CRISPR-Cas9 (Paix et al., 2016) or rescue of null mutants with GFP fusions expressed from transgenes, this approach enables routine protein depletion controlled by the spatial and temporal expression pattern of the promoter driving the GFP-degrading module. We describe a toolkit of transgenes expressing GFP degradation adaptors in different tissues that should facilitate tissue-specific analysis of protein function in *C. elegans*.

RESULTS

Epidermal expression of a GFP nanobody::ZIF-1 fusion depletes GFP-tagged proteins that localize to different subcellular locations

To selectively degrade GFP-tagged proteins, we expressed a GFP nanobody::ZIF-1 fusion under different tissue-specific promoters (Fig. 1A). This fusion acts as a GFP-to-ligase adapter that promotes

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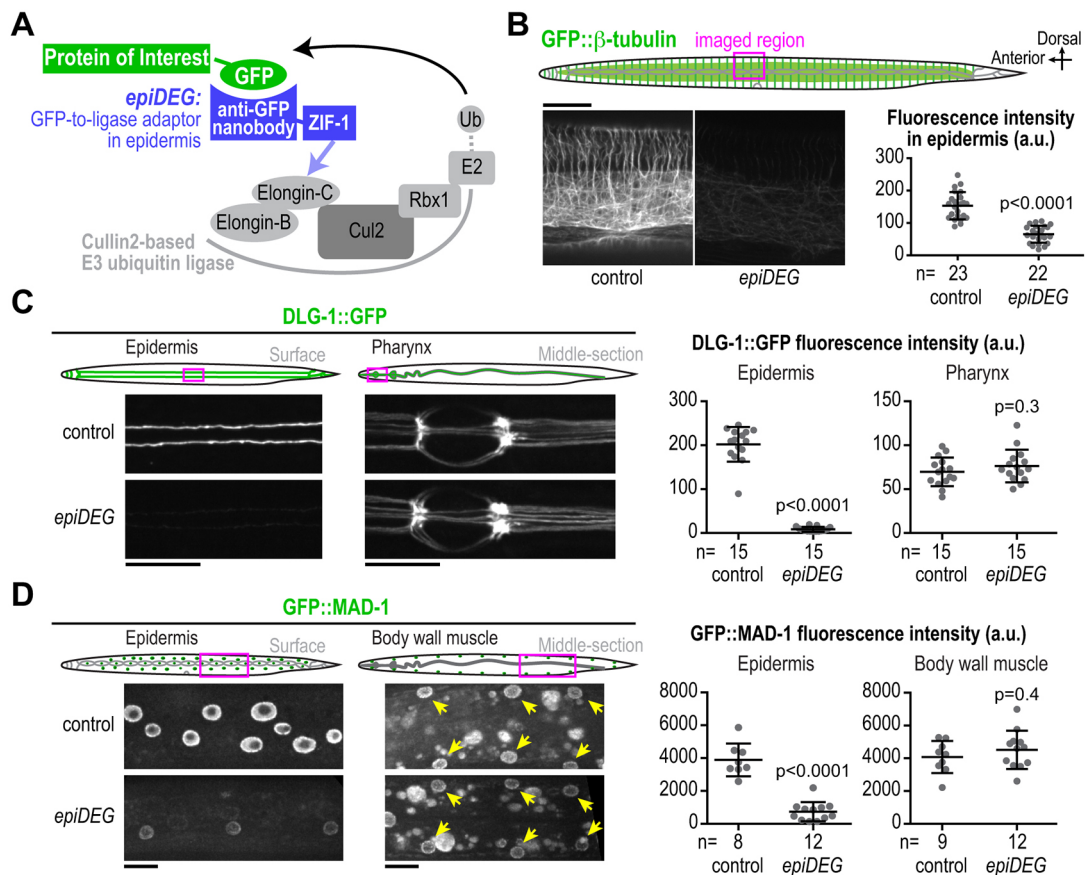


Fig. 1. *epiDEG* efficiently degrades GFP-tagged proteins that localize to different subcellular localizations. (A) Schematic illustrating the method. (B) Top: schematic showing imaged region. Bottom: fluorescence confocal images of L3 stage worms expressing GFP:: β -tubulin and plots of GFP:: β -tubulin fluorescence intensity. (C) Left: schematics and fluorescence confocal images of late L4 stage worms expressing DLG-1::GFP. Right: plots of DLG-1::GFP fluorescence intensity. (D) Left: schematics and fluorescence confocal images of L3 stage worms expressing GFP::MAD-1. Yellow arrows indicate the body wall muscle nuclei. Right: plots of GFP::MAD-1 fluorescence intensity. *n* is the number of worms analyzed. Data were analyzed using a two-tailed Student's *t*-test. *P* values are the probability of obtaining the observed results assuming the test group is the same as control. Data are shown as mean \pm s.d. Scale bars: 10 μ m.

ubiquitylation of the GFP-tagged protein by the Cul2 family E3 ligase CUL-2 and its subsequent degradation by the proteasome (DeRenzo et al., 2003). We have previously shown that epidermis-specific expression of a GFP nanobody::ZIF-1 fusion (*epiDEG*; Fig. 1A) led to efficient degradation of an endogenously tagged GFP fusion with the γ -tubulin complex component GIP-2 (Wang et al., 2015). To test whether *epiDEG* can target proteins that localize to different subcellular locations, we crossed the *epiDEG* transgene into strains expressing GFP-tagged proteins that localize to: the cytoplasm (transgene-encoded GFP:: β -tubulin expressed using the *dpy-7* promoter; Fig. 1B), apical cell junctions (transgene-encoded DLG-1::GFP expressed using the *dlg-1Δ7* promoter; Fig. 1C), and the nucleus and nuclear envelope [*in situ*-tagged GFP::MAD-1 (also called MDF-1 in the *C. elegans* literature); Fig. 1D]. Quantification (Fig. S1A-C) revealed a striking reduction in GFP fluorescence intensity in the larval epidermis for all three markers (DLG-1::GFP, 96%; GFP::MAD-1, 81%; GFP:: β -tubulin, 58%; Fig. 1B-D), whereas signal intensity was unchanged in control tissues (Fig. 1C,D; DLG-1::GFP and GFP::MAD-1, GFP:: β -tubulin was expressed in the epidermis only). We conclude that the GFP nanobody::ZIF-1 fusion can degrade proteins that localize to different subcellular locations; we note that it remains unclear whether depletion of proteins from the nucleus and cellular junctions occurs by targeting and degradation at these locations or by degrading the protein from the cytoplasm. With the exception of

GFP:: β -tubulin, which we expect is heavily expressed, reduction by *epiDEG* was consistently greater than 80% (Fig. 1C,D; Wang et al., 2015).

GFP-mediated protein degradation is efficient in multiple *C. elegans* tissues

To determine whether the GFP nanobody::ZIF-1 fusion could degrade GFP-tagged proteins in different tissues, we expressed the GFP nanobody::ZIF-1 fusion or ZIF-1 alone (as a control) using promoters that drive expression in the intestine (*intDEG*, *Pelt-2*; Fukushima et al., 1998), body wall muscle (*bwmDEG*, *Pmyo-3*; Fire and Waterston, 1989) and ciliated sensory neurons (*csnDEG*, *Posm-6*; Collet et al., 1998). The transgenes also included an operon linker (Huang et al., 2001) followed by an mCherry::Histone H2b reporter to allow identification of cells expressing the degradation module (*DEG*) or control transgenes (Fig. 2A). To assess the relative function of the degradation module in different tissues, the transgenes were introduced into a background expressing *in situ*-tagged GFP::MAD-1, which localizes to nuclei across differentiated tissues throughout development (Fig. S2A,B). In all three tested tissues, GFP::MAD-1 signal was eliminated when the GFP nanobody::ZIF-1 fusion, but not ZIF-1 alone, was expressed (Fig. 2B-D). We conclude that the GFP nanobody::ZIF-1 fusion targets GFP-tagged proteins for degradation in multiple tissues (promoters and targets are summarized in Table S1; an analysis

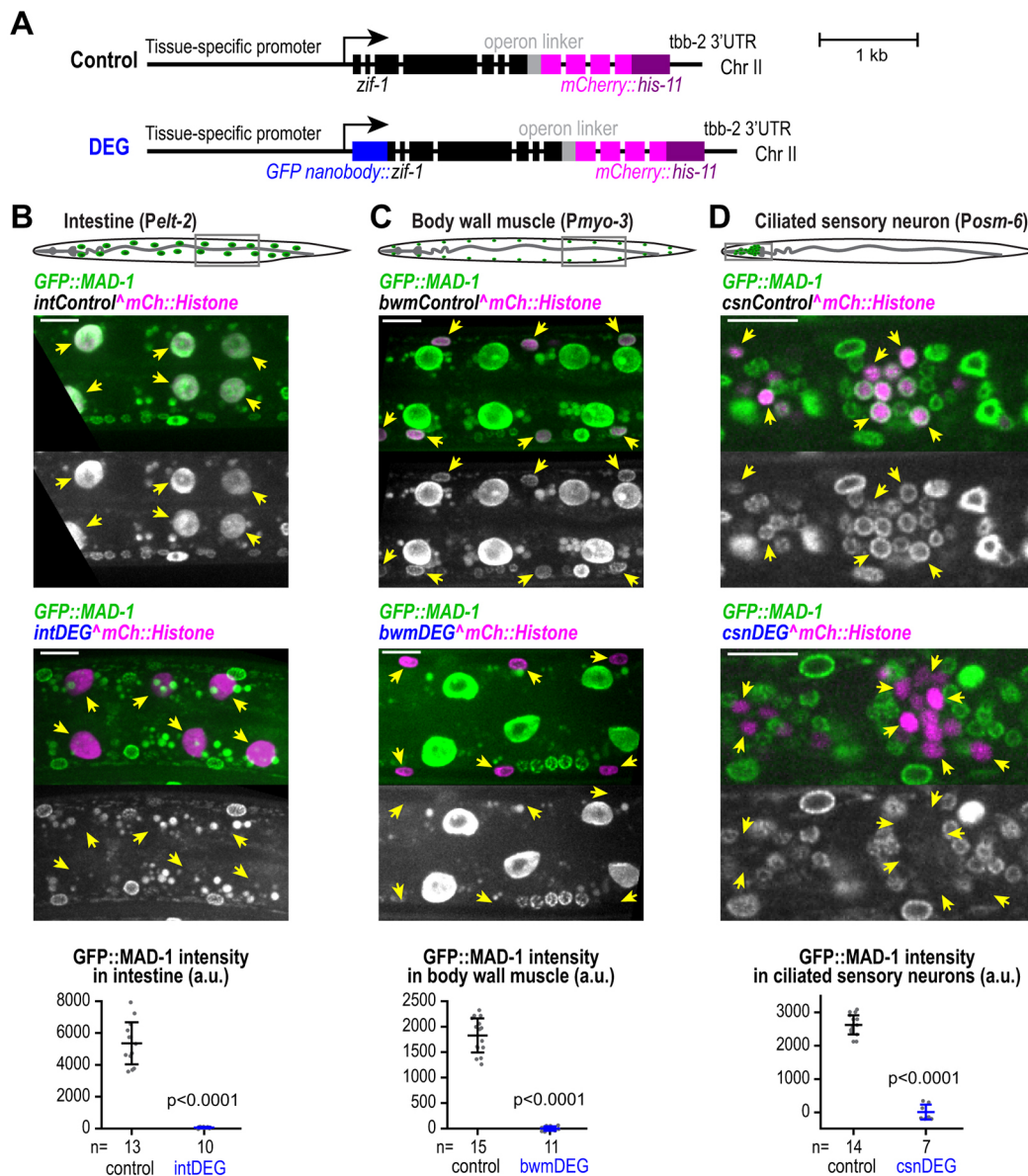


Fig. 2. GFP-mediated protein degradation is efficient in multiple *C. elegans* tissues. (A) Transgene schematics. (B–D) Top: schematics showing imaged region. Middle: fluorescence confocal images (maximum intensity projections in B and C, single z-slice in D) of L3 stage worms expressing GFP::MAD-1. Yellow arrows indicate nuclei. Bottom: plots of GFP::MAD-1 fluorescence intensity. *n* is the number of worms analyzed. Each data point in B and C represents an average of five nuclei from the same worm. Data were analyzed using a two-tailed Student's *t*-test. *P* values are the probability of obtaining the observed results assuming the test group is the same as control. Data are shown as mean \pm s.d. Scale bars: 10 μ m.

of reporter expression showing when each promoter turns on is in Fig. S3).

GFP-mediated protein degradation is rapid and target dependent

Next, we used time lapse imaging to follow degradation kinetics for two *in situ* GFP-tagged target proteins (GFP::MAD-1 and GFP::PP1^{GSP-2}) in the intestine of *C. elegans* embryos, where expression of the nanobody-ZIF-1 fusion is under the control of the *elt-2* promoter (*intDEG*). Degradation kinetics result from a combination of the kinetics with which levels of the nanobody-ZIF-1 fusion increase as the *elt-2* promoter becomes active and the time required for the fusion to shuttle the target for degradation. Thus, we would expect the degradation of more abundant proteins to take longer. Visual inspection suggested that GFP::MAD-1 was degraded within

~12 min (Fig. 3A and Movie 1). Owing to the relatively long folding time for the mCherry fluorophore, the GFP::MAD-1 signal had disappeared ~50 min before the first point where the mCherry::histone signal, which marks nuclei in cells expressing the *intDEG* cassette, could be detected (Fig. 3A and Movie 1). The rapid disappearance of GFP::MAD-1 prior to expression of our mCherry nuclear landmark prevented a more detailed quantification. The second target, GFP::PP1^{GSP-2}, is expressed at ~4-fold higher levels (Fig. 3B) and its cytoplasmic outline allowed us to quantify the signal back to degradation onset prior to appearance of the mCherry histone landmark. As for GFP::MAD-1, degradation of GFP::PP1^{GSP-2} began about 50 min before the point where the mCherry::histone signal first became detectable (Fig. 3C,D and Movie 2). However, in this case, the point when the signal was reduced by ~80% was about 30 min after the mCherry::histone signal first

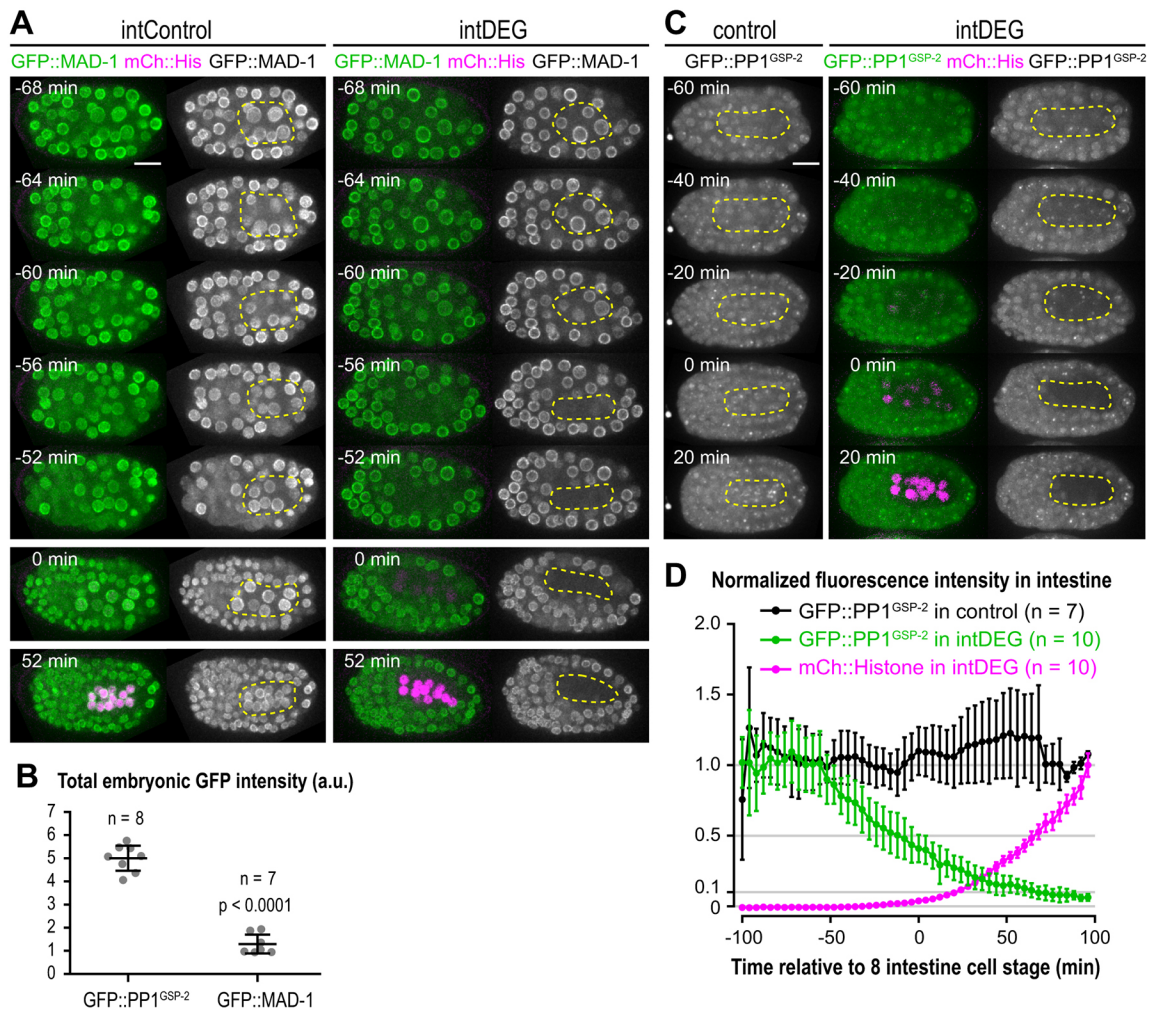


Fig. 3. GFP-mediated protein degradation is rapid in *C. elegans* intestine. (A) Still images from time-lapse confocal imaging of embryos expressing GFP::MAD-1 and either the *intControl* or *intDEG* cassettes (also see Movie 1). (B) Graph quantifying total per embryo GFP fluorescence for GFP::PP1^{GSP-2} and GFP::MAD-1. (C) Still images from time-lapse confocal imaging of control and intDEG embryos expressing GFP::PP1^{GSP-2} (also see Movie 2). Dashed yellow outlines show the location of the intestinal cells in A and C. (D) Plot of normalized GFP::PP1^{GSP-2} or mCherry::histone intensity in control or intDEG embryos. *n* is the number of embryos analyzed. Data are shown as mean±s.d. Scale bars: 10 μm.

became detectable (Fig. 3C,D and Movie 2). We conclude that penetrant degradation of both targets occurs rapidly relative to the time-scale of developmental events. However, the precise timing with which penetrant depletion is achieved following promoter turn-on depends on target-specific features, most likely abundance and/or accessibility.

Degradation of GFP::DLK-1 in the touch receptor neurons blocks axon regeneration

To determine whether GFP-mediated protein degradation could recapitulate loss-of-function phenotypes in the nervous system, we expressed the GFP nanobody::ZIF-1 fusion from a transgene that also included cytoplasmic mKate2 (to visualize axons) in the touch receptor neurons using the *mec-18* promoter (*trnDEG*; Fig. 4A). To assess efficacy, we targeted a GFP fusion with DLK-1 (Dual-Leucine zipper Kinase MAPKKK) (K. Noma and Y. Jin, unpublished), which is required to initiate axon regeneration (Hammarlund et al., 2009; Yan et al., 2009). Following laser-induced axotomy in the PLM touch neuron (Fig. 4B), GFP::DLK-1 promoted axon regrowth in the presence of endogenous DLK-1, consistent with the known effects of DLK-1 overexpression

(Hammarlund et al., 2009; Yan et al., 2009), while a *dlk-1* deletion mutant strongly impaired regrowth (Fig. 4C,D). GFP::DLK-1 expression fully rescued the impaired regrowth of the *dlk-1Δ* mutant, and this rescue was abolished by introduction of *trnDEG* (Fig. 4C-E). We note that no defects in axon regrowth were detected in *trnDEG* worms with endogenously tagged GIP-2::GFP or DHC-1::GFP (Fig. S4), suggesting that GIP-2 and DHC-1 are either not expressed or not required for axon regrowth in PLM touch neurons.

Degradation of GIP-2::GFP or PLK-1::sGFP in the intestine causes cell division defects and impairs *C. elegans* growth

To test whether we could use this method to circumvent complications associated with embryonic lethality and observe tissue-specific phenotypes, we analyzed the consequences of depleting two *in situ*-tagged targets, GIP-2::GFP (an essential component of the microtubule-nucleating γ-tubulin complex) and PLK-1::sGFP (fusion of PLK-1, a mitotic kinase that regulates cell cycle progression, with superfolder GFP) in the intestine. The *C. elegans* intestine arises from the E blastomere of the 8-cell embryo (Deppe et al., 1978). The *elt-2* promoter that drives *intDEG* expression turns on at the 2E stage (McGhee et al., 2007) and the

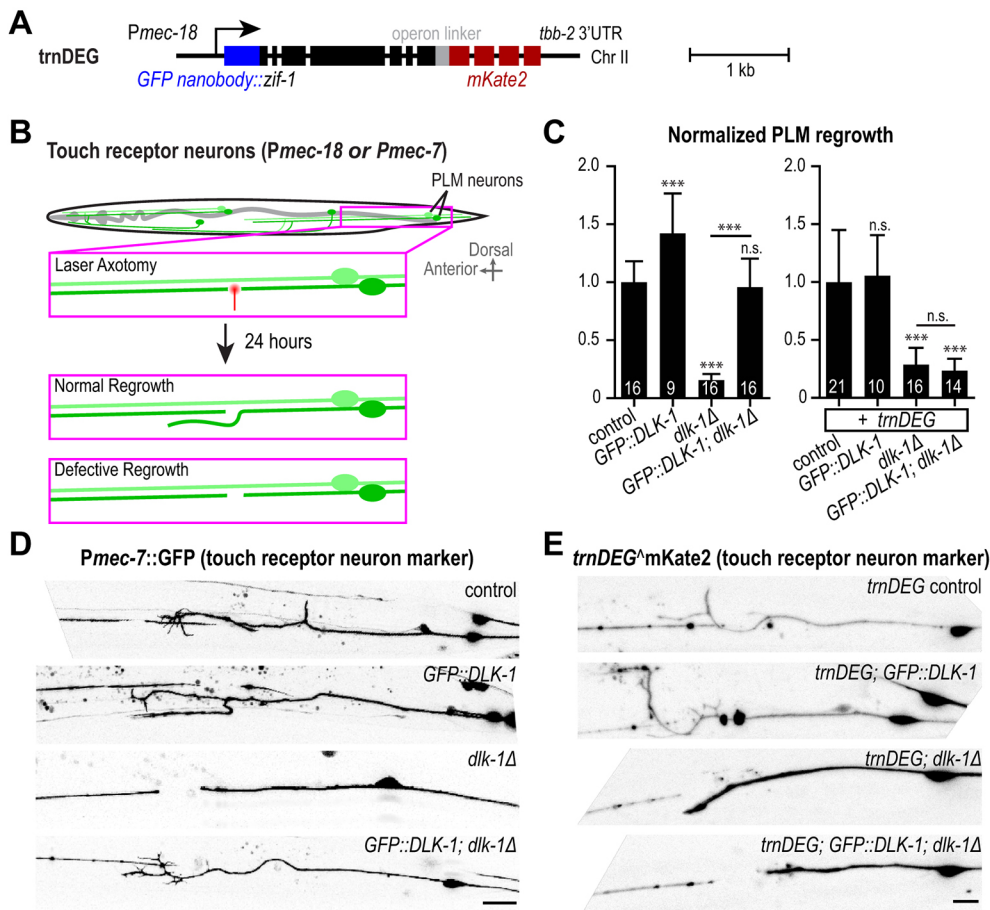


Fig. 4. Degradation of GFP::DLK-1 in the touch receptor neurons blocks axon regeneration. (A) Transgene schematic. (B) Schematic of the axon regeneration assay. (C) Plots of normalized touch neuron (PLM) regrowth at 24 h post laser axotomy. Number in each bar is the number of worms assayed. (D,E) Inverted grayscale images of the touch neuron (PLM) axon. Data were analyzed using a one-way ANOVA with Bonferroni's post test. *** $P < 0.001$. n.s., not significant. Results are compared with control unless specified by the line. Data are shown as mean \pm s.d. Scale bars: 10 μ m.

mCherry::Histone reporter becomes visible at the 8E stage (8-cell intestine; Fig. S3B). During intestinal differentiation in the embryo, the γ -tubulin complex re-localizes from centrosomes to the apical cell surface (Feldman and Priess, 2012; Fig. 5A). Co-expressing *intDEG*, but not the control transgene, eliminated the intestinal GIP-2::GFP and PLK-1::sGFP signals (Fig. 5A-C). As the γ -tubulin complex and PLK-1 are required for cell division (Hannak et al., 2002; Strome et al., 2001), we anticipated that their inhibition might reduce intestinal cell number. Consistent with a cell division defect, in 1.5- to 1.8-fold stage embryos, when control embryos typically have 20 intestinal cells, only 8-15 intestinal nuclei were detected in *intDEG* embryos with *in situ*-tagged GIP-2::GFP or PLK-1::sGFP (Fig. 5D); larval *intDEG* worms also grew more slowly and reached a smaller adult size than controls (Fig. 5E). Thus, intestinal degradation of GIP-2::GFP or PLK-1::sGFP not only resulted in a tissue-specific cell division defect consistent with their known functions, but also revealed a novel post-embryonic developmental defect, demonstrating the utility of the approach.

DISCUSSION

We describe a robust method for the degradation of GFP fusions that complements existing approaches – genetic locus removal, RNA interference, and auxin-, ZF1- and sortase-mediated degradation – to enable tissue-specific analysis of protein function in *C. elegans*. The utility of this approach is enhanced by the recent development of robust CRISPR/Cas9-based methods that enable routine GFP tagging of endogenous loci (Dickinson et al., 2013; Paix et al., 2016). We expect that the set of strains we describe here will be a useful resource to allow the tissue-specific degradation of this

emerging collection of *in situ*-tagged proteins and will serve as a template for engineering additional versions that will expand the utility of this strategy.

In principle, degradation kinetics will be influenced by the strength of promoter driving the degron cassette, the efficiency of CUL-2-dependent proteasomal degradation in the target tissue, and target abundance and/or accessibility. An analysis of degradation kinetics for two *in situ*-tagged targets, GFP::MAD-1 and GFP::PP1^{GSP-2}, in the embryonic intestine revealed that GFP::MAD-1 was degraded with a half-life of ~ 6 min, whereas GFP::PP1^{GSP-2}, which is more abundant and cytoplasmic/nucleolar rather than nuclear, was degraded with an ~ 40 min half-life. These degradation rates are comparable with rates published previously for ZF1 degron-tagged fusions targeted by ZIF-1 expression (15-30 min half-life; Armenti et al., 2014), auxin-inducible degradation (~ 20 min half-life; Zhang et al., 2015) and sortase-based F-box ligation-mediated degradation (~ 1 h half-life; Wu et al., 2017). Thus, degradation rates will differ between targets depending on abundance and accessibility, but are likely to be comparable for the different methods.

Of the described methods for conditional protein degradation in *C. elegans* (this report; Armenti et al., 2014; Wu et al., 2017; Zhang et al., 2015), each has advantages and limitations. The auxin-inducible degron enables simultaneous spatial (via tissue-specific expression of the TIR1 adaptor) and temporal (timing of auxin addition) control. However, this system requires tagging of the target with the AID degron and auxin addition as well as expression of the TIR1 adaptor in the target tissue. The sortase-based method uses the smallest tag (only 5 amino acids), which could minimize interference with protein function; however, degradation appears to be somewhat

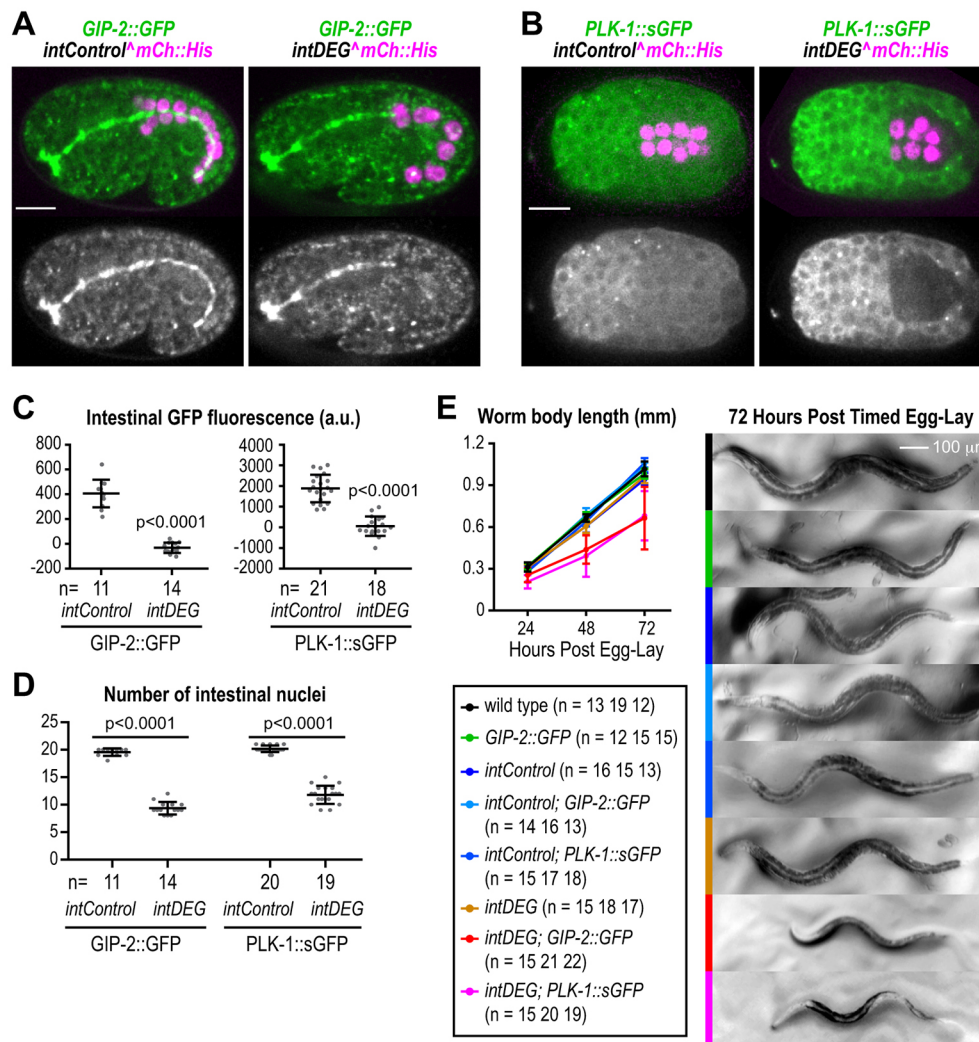


Fig. 5. Degradation of GIP-2::GFP or PLK-1::sGFP in the intestine causes cell division defects and impairs growth. (A) Maximum intensity projections of confocal images of 1.5-fold *C. elegans* embryos expressing GIP-2::GFP with the *intControl* or *intDEG* cassettes. (B) Confocal images of pre-comma *C. elegans* embryos expressing PLK-1::sGFP with the *intControl* or *intDEG* cassettes. (C) Plots of intestinal GFP fluorescence for GIP-2::GFP in 1.5- to 1.8-fold embryos (left) and PLK-1::sGFP in pre-comma embryos (right). *n* is the number of embryos analyzed. (D) Plot of the number of intestinal nuclei in 1.5- to 1.8-fold embryos. (E) Left: plot of body length for worms of the indicated genotypes. Right: representative images of worms at 72 h post timed egg-lay. *n* is the number of worms analyzed at 24, 48 and 72 h. Data were analyzed using a two-tailed Student's *t*-test. *P* values are the probability of obtaining the observed results, assuming the test group is the same as control. Data are shown as mean±s.d. Scale bars: 10 μm (or as indicated).

slower with lower penetrance. Both the 36 amino acid ZF1 degnon (Armenti et al., 2014) and the 44 amino acid AID (Zhang et al., 2015) tags are significantly smaller than GFP (238 amino acids) and thus less likely to interfere with protein function. However, since having a fluorescent tag on the target protein is useful for monitoring its degradation, most reported ZF1 and AID degnons to date have been added in conjunction with a GFP tag (Armenti et al., 2014; Zhang et al., 2015). As the CRISPR-Cas9-mediated knock-in method becomes increasingly popular, *in situ* tagging with GFP to enable monitoring of protein localization is likely to become commonplace, and an advantage of our method is that it will allow tissue-specific degradation of these proteins, without any additional engineering, by simply crossing in the transgene that expresses the GFP nanobody::ZIF-1 fusion in the tissue of interest.

A potential concern for both the GFP- and ZF1-mediated degradation methods is that ectopic expression of ZIF-1 or the GFP nanobody::ZIF-1 fusion could lead to off-target effects. Endogenous ZIF-1 is transiently expressed in somatic cells to remove some germline-specific proteins (e.g. PIE-1; DeRenzo et al., 2003). Consistent with the idea that its natural targets are limited to the germline, expressing ZIF-1 in all somatic tissues using a *cdc-42* promoter (Armenti et al., 2014) or the GFP nanobody::ZIF-1 fusion in five different somatic tissues (our data) has not led to any obvious developmental defects. Therefore, ZIF-1-based protein degradation approaches are likely compatible with all somatic tissues. However,

as ZIF-1 naturally targets proteins, including PIE-1, that function in the germline, methods that rely on expression of ZIF-1 (Armenti et al., 2014) or a GFP nanobody::ZIF-1 fusion (this report) are likely incompatible with use in the germline.

The GFP nanobody we use here (vhhGFP4; Rothbauer et al., 2006) recognizes common GFP variants such as EGFP, Venus, YFP, EYFP (Caussinus et al., 2012) and superfolder GFP (Fig. 5B,C), but not coral-derived red fluorescent proteins. We have preliminary data that the nanobody also does not recognize mNeonGreen (D.K.C., unpublished), a lancelet-derived green fluorescent protein distantly related to Aequorea GFP (Shaner et al., 2013) that exhibits robust fluorescence in *C. elegans* (Dickinson et al., 2015). Thus, red fluorescent proteins are a good choice for marker fusions for phenotypic analysis in the presence of the degnon; mNeonGreen could also be used in cases where it is not necessary to use the green channel to monitor degradation of the tagged GFP fusion.

MATERIALS AND METHODS

C. elegans strains

C. elegans strains (listed in the supplemental Materials and Methods) were maintained at 20°C. Transgenic strains were engineered as described previously (Dickinson et al., 2013; Frøkjær-Jensen et al., 2008). Briefly, GFP or sGFP was fused to the N terminus of MAD-1 and PP1^{GSP-2}, and the C terminus of GIP-2, PLK-1 and DHC-1 at their endogenous loci using CRISPR-Cas9 (Dickinson et al., 2013). Transgenes encoding C-terminally

tagged DLG-1::GFP, N-terminally tagged GFP:: β -tubulin^{TBB-2} and GFP::DLK-1 were generated using Mos1 transposon-mediated single-copy insertion (Frøkjær-Jensen et al., 2008). The DEG transgenic strains will be made available through the *Caenorhabditis* Genetics Center (CGC).

Plasmid construction

Plasmids were constructed using Gibson Assembly (Gibson et al., 2009). Briefly, the GFP nanobody vhhGFP4 fragment was PCR amplified from pcDNA3_NSImb-vhhGFP4 (Addgene # 35579); the ZIF-1 genomic DNA (from ATG to TAA) and tissue-specific promoters were PCR amplified from N2 genomic DNA; the operon linker, mCherry::Histone H2b reporter and *ttb-2* 3'UTR were amplified from a previous construct in the lab (pOD1248); and the MosSCI targeting vector backbone was amplified from pCFJ151 (Frøkjær-Jensen et al., 2008). PCR primers were designed so that neighboring DNA fragments in the same construct had at least 20 bp overlap at each junction. Equimolar DNA fragment mixture (5 μ l) was mixed with 15 μ l of 1.33 \times home-made Gibson Assembly mix [133 mM Tris-HCl (pH 7.5), 267 μ M each dNTP, 1.33 mM NAD, 6.67% PEG 8000, 13.3 mM MgCl₂, 13.3 mM DTT, 5.33 U/ml Epicentre T5 exonuclease, 33.3 U/ml Phusion high-fidelity DNA polymerase and 5.33 U/ μ l Taq DNA ligase], and incubated at 50°C for 1 h before 5-10 μ l of the reaction was used for transformation. The DEG plasmid constructs along with complete sequences and maps have been deposited in Addgene by K.O. (Plasmid IDs 89357, 89366, 89367, 89368 and 89369).

Laser axotomy and light microscopy

Laser axotomy was performed as described previously (Chen et al., 2011). Images in Fig. 1B and Fig. S1A were acquired using an inverted Zeiss Axio Observer Z1 system equipped with AxioVision software, a Yokogawa spinning-disk confocal head (CSU-X1), a 63 \times 1.40 NA Plan Achromat lens (Zeiss) and a Hamamatsu ORCA-ER camera (Model C4742-95-12ERG, Hamamatsu Photonics). Images in Figs 1C,D, 5A and Fig. S1B were acquired on the same system using an EMCCD camera (QuantEM:512SC, Photometrics). Images in Fig. 2, Figs S1C-D and S2 were acquired using a Nikon TE2000-E inverted microscope equipped with Andor iQ2 software, a Yokogawa spinning-disk confocal head (CSU-10), a 60 \times 1.40 NA Plan Achromat lens (Nikon, Tokyo, Japan) and an EMCCD camera (iXon DV887ECS-BV, Andor Technology). Images in Fig. 3A,C, Fig. 5B, Fig. S3, and Movies 1-2 were acquired using a CV1000 spinning disk confocal system (Yokogawa Electric) maintained in a 16°C temperature-controlled room, using a 60 \times 1.35NA U-PlanApo objective and a 512 \times 512 EM-CCD camera (Hamamatsu). Images in Fig. 4D,E and Fig. S4 were acquired using Zeiss LSM510 (Fig. 4D) and LSM710 (Fig. 4E and Fig. S4; Zeiss Plan Achromat 63 \times 1.4 NA oil DIC objective) confocal microscopes controlled by ZEN software (Zeiss). Images in Fig. 5E were acquired using the DinoEye eyepiece camera (AM7023B, Dino-Lite) mounted on a Nikon SMZ800 dissection scope using the DinoXcope software (Dino-Lite).

Image analysis

Image analysis was first performed with Fiji (Image J) in a semi-automated manner aided by customized macros. Either a box or a line was made inside or across the region of interest to measure raw GFP intensities. Raw measurements were analyzed using customized Python scripts to compute final values. For details, see Fig. S1 and the supplementary Materials and Methods.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: S.W., N.H.T., A.D.C., A.D., K.O.; Methodology: S.W.; Formal analysis: S.W., N.H.T., P.L.-G.; Investigation: S.W., N.H.T., P.L.-G., Z.Z., D.K.C.,

B.P.; Writing - original draft: S.W., A.D., K.O.; Writing - review & editing: S.W., N.H.T., A.D.C., A.D., K.O.; Visualization: S.W., N.H.T.; Supervision: A.D.C., K.O.; Project administration: A.D.C., A.D., K.O.; Funding acquisition: A.D.C., A.D., K.O.

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Supplementary information

Supplementary information available online at <http://dev.biologists.org/lookup/doi/10.1242/dev.150094.supplemental>

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